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COMPARISON OF GLYCOSYLATED AND DEGLYCOSYLATED VITELLINS FROM FOUR SPECIES OF WEEVIL (COLEOPTERA: CURCULIONIDAE)

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Abstract—1. The vitellins or yolk proteins of four species of weevil (Coleoptera: Curculionidae) consist of two size classes with molecular weights of 150–170 and 42–47 kilo daltons (kDa).

2. The boll weevil, *Anthonomus grandis*, has one large vitellin while *Smicronyx fulvus*, *S. sordidus*, and *Cylindrocopturus adspersus* each have two large vitellins.

3. All four weevils have a single small vitellin whose size differs with genera.

4. In all four species both large and small vitellins contain *N*-linked glycosyl groups.

5. Enzymatic removal of the *N*-linked glycosyl chains lowers the molecular weight of the vitellins 9–17%.

INTRODUCTION

The vitellins or yolk proteins are among the most abundant proteins made by egg-laying animal species. Insect viellins are produced principally by the female fat body, secreted to the hemolymph and then reabsorbed by the ovaries and deposited in the developing oocyte. There, they form into yolk platelets which, after fertilization, will become the principal nutritive source of the developing embryo (see reviews by Kunkel and Nordin, 1985; Byrne *et al.*, 1989; Raikhel and Dhadialla, 1992).

Three principal types of vitellin have been classified in insects (Harnish and White, 1982a). Class I vitellins consist of large and small proteins. This is the most common class and is found among Lepidoptera, Coleoptera, Orthoptera and lower Diptera. Class II consists of only a single large protein and is found in the Hymenoptera, most notably in the honey bee *Apis mellifera*. Class III vitellins consist of from 2 to 5 small proteins with no large protein. This class is found only among some higher dipterans. Because *Drosophila melanogaster* has the class III yolk proteins they have been the most studied class. However, the *Drosophila* yolk proteins show little, if any, structural or sequence similarity to other vitellins and, in fact, have been shown to be related to a lipase gene family (Terpstra and AB, 1988).

It has been shown by a number of methods, including *in vivo* pulsed labeling (Della-Cioppa and Engelmann, 1987; Dhadialla and Raikhel, 1990), cell-free translation (Bose and Raikhel, 1988) and monoclonal antibodies (Raikhel and Bose, 1988; Dhadialla and Raikhel, 1990) that the two proteins of the group II vitellins are probably derived from a single precursor vitellogenin by proteolytic cleavage such as occurs with vertebrate vitellogenins. Besides the proteolytic cleavage of the precursor, the vitellins are extensively glycosylated (Kunkel and Nordin, 1985; Dhadialla and Raikhel, 1990) and also modified by the addition of lipids (Shapiro *et al.*, 1988), phosphate (Della-Ciopa and Engelmann, 1987) and sulfate (Dhadialla and Raikhel, 1990). In some lepidopterans, it is reported that only the large vitellin is glycosylated (Mundall and Law, 1979; Susumi *et al.*, 1980).

Most studies of insect vitellins have concentrated on the locusts, Diptera and cockroaches. Less is known about the vitellins from the Coleoptera. *Tenebrio molitor*, the yellow mealworm (Harnish and White, 1982b) and the seven-spotted lady-beetle, *Coccinella septempunctata* (Okuda and Chinzei, 1988) have been shown to each have several large and small vitellins, placing them both in class I. Recently, Trewitt *et al.* (1992) have also investigated the vitellins of a curculionid beetle, the boll weevil, *Anthonomus grandis* Boheman, by isolating and sequencing the entire gene for the precursor vitellogenin.

I report here a comparison of the vitellin proteins, glycosylated and deglycosylated, of four species of weevil (Curculionidae), all serious pests of agricultural crops. *A. grandis*, the cotton boll weevil is a serious pest of the cotton crop. Larvae of *Cylindrocopturus adspersus* LeConte, the sunflower stem weevil feed in the stalk of the sunflower plant weakening or breaking it (Barker *et al.*, 1989). *Smicronyx fulvus* LeConte, the red sunflower see weevil (Oseto and Braness, 1979) and *Smicronyx sordidus* LeConte, the

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gray sunflower weevil (Brewer, 1991) feed on the developing seed kernel, seriously reducing crop yields.

MATERIALS AND METHODS

Boll weevils of the ebony strain (Bartlett, 1967) and sunflower stem weevils were reared on artificial diet (Barker *et al.*, 1989). Eggs were collected after an overnight laying period. The red and grey sunflower seed weevils were collected from sunflower heads growing in test plots near Prosper, ND. Females were dissected and mature oocytes collected.

Eggs of all species were homogenized in 0.05 M Tris–HCl, pH 6.8, in a glass Dounce homogenizer. The homogenate was centrifuged at 3000 *g* for 5 min and the aqueous layer separated from insoluble debris and an oil and lipid layer on top. This extract was used directly for electrophoretic analysis and enzymatic digestion. Protein concentrations were measured by the method of Bradford (1976).

Glycosidase digestions were performed in 25 μl containing 3–5 μg of yolk protein, 0.2 M Na_3PO_4, pH 7.2, 10 mM EDTA, 0.1% SDS, 1% octylglucoside, 1% mercaptoethanol, and 0.5 unit of endoglycosidase F, (E C 3.2.1.96) (Boehringer-Mannheim, Germany). This was incubated at 37°C for 12–16 hr.

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed on digested samples and the gels were stained with Coomassie Brilliant Blue. Molecular weights of the vitellin bands were measured in relation to known marker proteins. To accurately measure the molecular weight of the large vitellin proteins, 7% acrylamide gels were allowed to run for longer periods of time in order to provide better resolution of the bands.

RESULTS

Figure 1 shows the pattern obtained on SDS–PAGE gels of egg extracts of each of the four weevil species. In all cases, at least 90% of the water-soluble protein of the eggs is made up of two to three size classes. The boll weevil yielded two principal bands with molecular weights of 160 and 47 kDa. The three other species showed a similar large and small pattern but exhibited differences in size and number of bands. In all cases, the measured molecular weights of the large vitellins are the average of at least three different gels containing less protein than those lanes shown in Fig. 1, and run for longer periods of time in order to more clearly separate the high molecular weight bands.

Both *Smicronyx* species appear to be the same. Their single small vitellin is approximately the same size as the small boll weevil vitellin. However, each appears to possess two high molecular weight bands, one of them slightly larger than the single boll weevil band. The larger of the two, measured at 175 kDa, is consistently much more abundant than the slightly smaller 160 kDa protein.

The vitellins of the sunflower stem weevil exhibit the most differences in comparison with the boll weevil. The single small vitellin forms a sharp band at 43 kDa, significantly smaller than the 47 kDa of the other three species. Again there are two high molecular weight bands, 170 and 155 kDa, both of slightly different molecular weights than the vitellins of the other three species. In this species the smaller of the two large vitellins is the more abundant.

The vitellins of all species so far studied contain carbohydrate groups (Kunkel and Nordin, 1985). To investigate the glycosylation of the weevil vitellins I enzymatically deglycosylated them and compared the sizes of the deglycosylated and native proteins by SDS–PAGE. Extracts of eggs from all four weevil species were treated with *N*-glycosidase F, an enzyme that removes *N*-linked carbohydrate side-chains from protein. The results of this experiment are shown in Fig. 2.

All four weevil egg extracts showed a decrease in molecular weight of all vitellin proteins. The amount

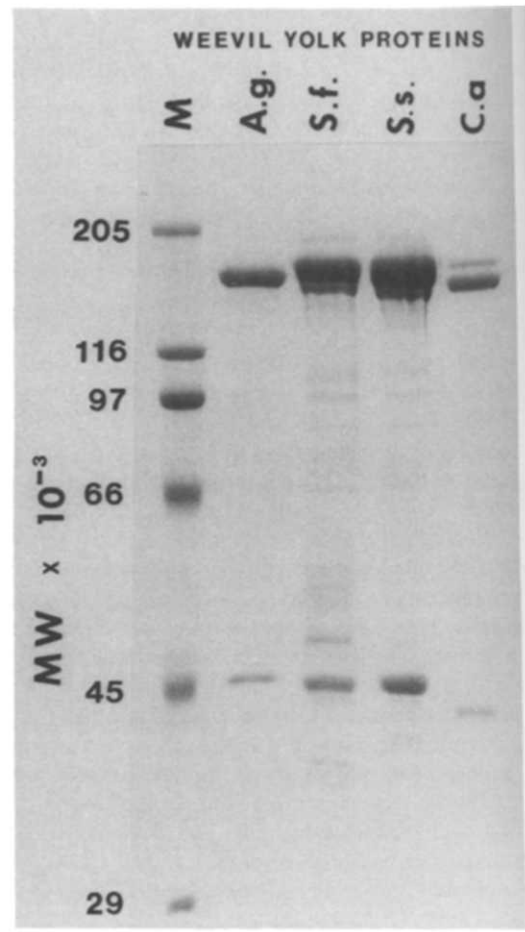

Fig. 1. SDS gel of the vitellin proteins of four species of weevil. M—Molecular weight marker proteins; Ag—*A. grandis*; Sf—*S. fulvus*; Ss—*S. sordidus*; Ca—*C. adspersus*.

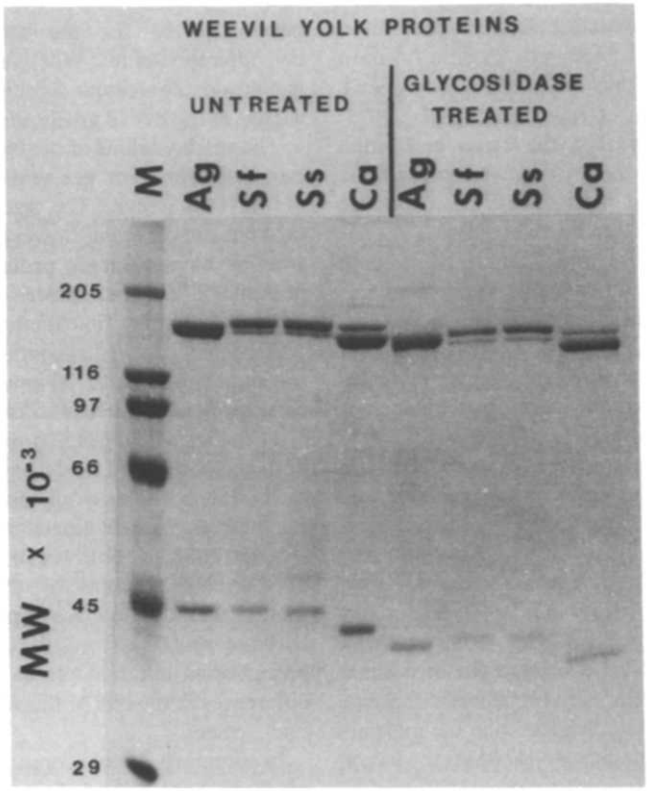

Fig. 2. SDS gel of the vitellin proteins of four species of weevil before and after treatment with *N*-glycosidase F. M—Molecular weight markers; Ag—*A. grandis*; Sf—*S. fulvus*; Ss—*S. sordidus*; Ca—*C. adspersus*.

of change was not the same in all cases, however. The major change in pattern was among the small vitellins. In the native state the boll weevil and the two sunflower seed weevils, *S. fulvus* and *S. sordidus*, have small vitellins of approximately 47 kDa. After deglycosylation, the boll weevil vitellin was slightly smaller, 39 vs 41 kDa. Both *Smicronyx* species decreased in size but were indistinguishable from each other. The *Cylindrocopturus* small vitellin changed from 43 to 36 kDa. The small vitellins appear to be 13-17% *N*-linked carbohydrate. All large vitellins of each weevil species also decrease in size upon deglycosylation, but only 9–10%. All the large vitellins appear to decrease in molecular weight about the same amount, 15 kDa. Table 1 summarizes the number and size of vitellin proteins of each species.

Table 1. Measured molecular weights ($\times 10^{-3}$) of the weevil vitellin proteins before and after *N*-glycosidase F treatment

	Untreated				Glycosidase-treated			
	Ag	Sf	Ss	Ca	Ag	Sf	Ss	Ca
HMW	160	175	160	170	145	160	160	155
		160	145	155		145	145	140
LMW	47	47	47	43	39	41	41	36

Ag—*A. grandis*; Sf—*S. fulvus*; Ss—*S. sordidus*; Ca—*C. adspersus*.

DISCUSSION

A wide array of actions and interactions makes the vitellins important items of study in many areas of cell and developmental biology. For the same reasons, and because of their critical importance in reproduction, they are potentially valuable for new methods of pest control (Whitten, 1989; Eggleston, 1991). Such methods, based on genetics, could target specific species by, for instance, allowing the generation of male-only lines for sterile male release by attaching the female-specific promoter of the vitellogenin gene to a toxin gene that, upon expression, would kill all females. It is also possible to envision specifically disrupting the reproductive processes of the insect. With their large number of molecular and cellular interactions, vitellins are a prime candidate for use in these methods. To reach this point we need much more data about the molecular and cell biology of the vitellins and their genes.

Only recently have there been attempts to extend the vitellin protein studies to the gene level. Genes from the locust (Locke *et al.*, 1987) and *Aedes* (Gemmill *et al.*, 1986) and *Anopheles* (Romans, 1989) mosquitoes have been cloned and partially characterized. The only non-drosophilid insect vitellogenin gene to be completely sequenced is that of the boll weevil (Trewitt *et al.*, 1992). This gene is single-copy and shows considerable homology to the vitellogenin

genes of nematodes and vertebrate. It codes for a protein of 205 kDa, large enough to account for both the large and small vitellin proteins of the boll weevil. Amino acid sequencing (Heilmann *et al.*, 1993) has shown conclusively that the large and small vitellin proteins of the boll weevil are indeed the proteolytically processed products of a single large precursor protein coded for by this gene.

As no other data were available on the vitellin proteins of other curculionids I initiated a comparative study of the boll weevil and three other weevils, all prominent agricultural pests of the cultivated sunflower. Comparison of these should give indicates of variability and similarity in proteins and genes among a small group of closely related insects.

All four of the coleopteran species examined here are similar in having the great majority of the egg protein consisting of two or three vitellin proteins. The boll weevil is known to have one large and one small vitellin protein, both derived from a single precursor protein (Trewitt *et al.*, 1992; Heilmann *et al.*, 1993). All three of the other weevils examined here have at least two large vitellins. Since the difference in molecular weight between the two larger vitellins (15 kDa) is much less than the molecular weight of the small vitellins (43–47 kDa) the larger cannot be the unprocessed precursor.

The most plausible explanation for this is that there are at least two vitellogenin genes coding for slightly different-sized proteins. Since each of the weevil species has only a single size class of small vitellin, all of the size difference between the two large vitellins of each species must be in that portion of the precursor vitellogenin forming the large vitellin. The presence of more than one vitellogenin gene is not unusual. Indeed, the single copy boil weevil gene is an exception. The locust has two genes (Locke *et al.*, 1987) while *Aedes* mosquitoes are reported to have five (Gemmill *et al.*, 1986) and *Anopheles* mosquitoes three (Romans, 1989).

In the two *Smicronyx* species and in *Cylindrocopturus* there are distinct differences in the relative amounts of protein present in each of the two large vitellins. If there are two genes they must be expressed at different levels. Alternatively, the more abundant protein band could be composed of several different proteins of the same size, each coded by a different vitellogenin gene.

I have shown here, conclusively, that in all four species of weevil both size classes of vitellins contain *N*-linked carbohydrate groups. Thus, the weevils appear to differ from at least two species of Lepidoptera, *Bombyx mori* and *Manduca sexta*, both of which also have class I vitellins, but in which it is reported that only the larger vitellin is glycosylated (Mundall and Law, 1979; Susumi *et al.*, 1980). The carbohydrate appears to make up 9–17% of the native molecular weight of the proteins. This is a higher percentage than reported for other insects (1–11%) (Kunkel and Nordin, 1985). No figures have been reported for the carbohydrate content of coleopteran vitellins. Whether lipids or other modifying groups contribute significantly to the molecular weight of the weevil vitellins is not known at this time.

The small vitellins of the two *Smicronyx* species are essentially the same size as the corresponding protein of the boll weevil. The size of the deglycosylated proteins are different, however. The *Smicronyx* small vitellins have a larger protein core and a smaller percentage of carbohydrate. From the size difference, one would predict that the portion of the *Smicronyx* vitellogenin precursor containing the small vitellin sequence would be 15–20 amino acids longer than the corresponding sequence in the boll weevil precursor.

The small vitellin of *Cylindrocopturus* is smaller in both the native and deglycosylated state than that of the boll weevil. This would also indicate a smaller size for the precursor vitellogenin unless the large vitellin is larger than the boll weevil large vitellin. Since one of the large vitellins of *Cylindrocopturus* is also smaller than the boll weevil protein, it seems unlikely to have resulted from the cutting of a same-size precursor in different places. More likely, there are differences in the size of the vitellogenin precursors of each species.

In summary, I have compared the vitellin proteins of three genera and four species of curculionid Coleoptera. While all have large and small vitellins there were differences in the number and size of the proteins, particularly the large vitellins. Both large and small vitellins of all the species are heavily glycosylated, containing 9–17% *N*-linked carbohydrate. Comparison of the sunflower weevils with the better characterized boll weevil vitellins indicated possible differences in the number of vitellogenin genes and the size of the vitellogenin precursors.

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